SECOND EDITION

IMMUNOLOGY

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Second Edition

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W. H. Freeman and Company
New York

Cover credits
Background: lymph node macrophage attached to an endothelial cell.

Inset: X-ray crystallography of a peptide bound to a human class II MHC molecule, DR1.
Courtesy of J. H. Brown, 1993, *Nature* 364:33.

Library of Congress Cataloging-in-Publication Data

Kuby, Janis.
Immunology / Janis Kuby. — 2nd ed.
p. cm.
Includes bibliographical references and index.
ISBN 0-7167-2400-6
1. Immunology. I. Title.
[DNLM: 1. Immune System. 2. Immunity. QW 504 K951 1994]
QR181.K83 1994
616.07'9—dc20
DNLM/DLC
for Library of Congress 93-46423
CIP

Printed in the United States of America

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TABLE 4-4 COMPARISON OF ANTIGEN RECOGNITION BY T CELLS AND B CELLS

Characteristic	B cells	T cells
Interaction with antigen	Involves binary complex of membrane Ig and Ag	Involves ternary complex of T-cell receptor, Ag, and MHC molecule
Binding of soluble antigen	Yes	No
Involvement of MHC molecules	None required	Required to display processed antigen
Chemical nature of antigens	Protein, polysaccharide, lipid	Only protein
Epitope properties	Accessible, hydrophilic, mobile, often nonsequential (conformational) peptide	Internal, denatured, amphipathic, linear peptide that can bind to MHC molecule

Other adjuvants, such as synthetic polyribonucleotides and bacterial lipopolysaccharides, stimulate nonspecific lymphocyte proliferation and thus increase the likelihood of antigen-induced clonal selection of lymphocytes. Some adjuvants stimulate a local, chronic inflammatory response with an increase in phagocytic cells as well as lymphocytes. This cellular infiltration at the site of the adjuvant injection can often result in a dense, macrophage-rich mass of cells called a *granuloma.* Both alum and Freund's complete and incomplete adjuvants cause granuloma formation. The increased numbers of phagocytic cells at the site of the granuloma are thought to facilitate antigen processing and presentation and may also increase production of interleukin 1, thus stimulating activation of T_H cells.

An additional mechanism of adjuvant action has been revealed by more recent experiments. When a T_H cell recognizes antigen associated with a class II MHC molecule on the membrane of an antigen-presenting cell, the T_H cell needs a second signal, called a *co-stimulatory signal,* to become activated. One such co-stimulatory signal is generated by the interaction between two membrane molecules: B7, present on macrophages, and CD28, present on T_H cells. When antigen is injected with complete Freund's adjuvant, macrophages increase their expression of the B7 membrane molecule. Thus, the requisite co-stimulatory signal may be generated more easily in the presence of adjuvant than in its absence.

EPITOPES

As mentioned in Chapter 1, immune cells do not interact with, or recognize, an entire immunogen molecule; instead, lymphocytes recognize discrete sites on the macromolecule called *epitopes,* or *antigenic determinants.* Epitopes are the immunologically active regions of an immunogen that bind to specific membrane receptors for antigen on lymphocytes or to secreted antibodies. Interaction between lymphocytes and a complex antigen may involve several levels of antigen structure. In the case of protein antigens, the structure of an epitope may involve elements of the primary, secondary, tertiary, and even quaternary structure of the protein (see Figure 4-1). In the case of polysaccharide antigens, extensive side-chain branching via glycosidic bonds affects the overall three-dimensional conformation of individual epitopes.

T cells and B cells exhibit fundamental differences in antigen recognition (Table 4-4). B cells recognize soluble antigen when it binds to their membrane-bound antibody. Because B cells bind antigen that is free in solution, the epitopes they recognize tend to be highly accessible sites on the exposed surface of the immunogen. Such exposed epitopes generally contain hydrophilic amino acids and are often located at bends in the amino acid chain, imparting a greater degree of mobility to these residues. T cells, on the other hand, recognize processed peptides associated with MHC molecules on the surface of antigen-presenting cells and altered self-cells. T cells thus exhibit *MHC-restricted antigen recognition.* The CD4 subpopulation recognizes antigen in association with class II MHC molecules and generally functions as T helper cells, whereas the CD8 subpopulation recognizes antigen in association with class I MHC molecules and generally functions as T cytotoxic cells. The CD4 cell is therefore said to be class II restricted and the CD8 cell is said to be class I restricted. Subtle differences in the class I or class II MHC molecules expressed by different individuals influence their ability to recognize T-cell epitopes. Thus T-cell epitopes cannot be considered apart from their associated MHC molecules.

Determination of the conformation of an epitope is a time-consuming task requiring knowledge of its primary sequence and often of its three-dimensional structure, as well as information on the immune reactivity of each region of that structure. Some T-cell and B-cell epitopes have been identified by a technique called epitope mapping. In this technique, an immunogenic protein is fragmented with proteolytic enzymes into overlapping peptides, which are then tested for their ability to bind to an antibody elicited by the native protein or to induce T- or B-cell activation. This approach has been particularly useful in mapping T-cell epitopes since the T cell recognizes short linear peptides complexed with MHC molecules. This method is less effective for determining B-cell epitopes, which are often not contiguous amino acid sequences but instead are brought together in the tertiary folded configuration of the protein. These conformational B-cell epitopes cannot be identified by epitope mapping.

In a few cases B-cell epitopes have been identified by x-ray crystallographic analysis of Ag-Ab complexes. In this procedure beams of x-rays are passed through a crystal of an Ag-Ab complex. This analysis generates a three-dimensional space-filling model of every atom in the complex, allowing identification of the epitope and the contact residues of the antibody's antigen-binding site. Analysis of the x-ray diffraction patterns is extremely complex and takes years to complete. Consequently, only a few Ag-Ab complexes have been analyzed by this method. Needless to say, detailed understanding of epitope structure has not been attained for most immunogens.

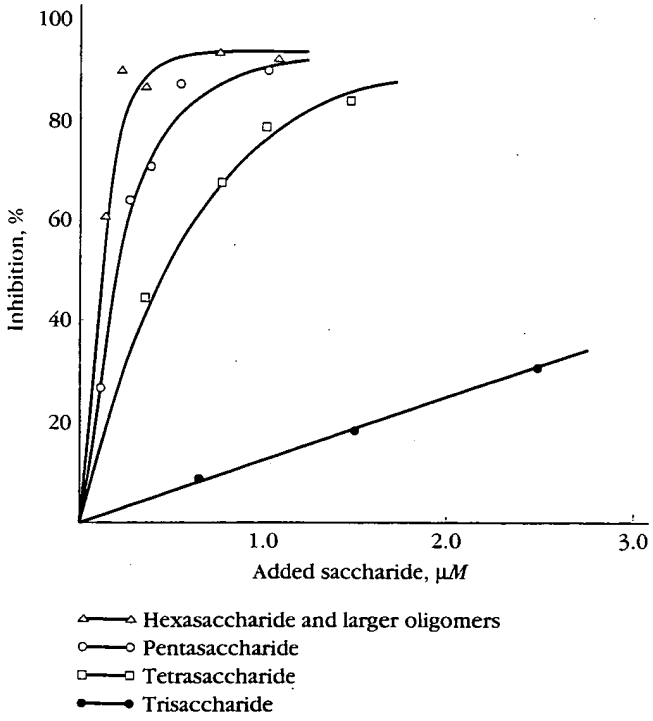


FIGURE 4-3 Ability of glucose oligomers of various sizes to inhibit the dextran-antidextran reaction. Rabbits were immunized with the glucose polymer dextran, and the antidextran antibodies produced were isolated and incubated with various short oligomers. Binding of the antidextran antibodies to dextran then was determined. Since the greatest inhibition would be expected with oligomers that bound most effectively to the dextran-binding site on the antibody molecules, these data can be used to estimate the size of the dextran epitope recognized by B cells. [Data from E. Kabat, 1974, *J. Am. Chem. Soc.* **76**:3709.]

Properties of B-Cell Epitopes

Several generalizations have emerged about properties of B-cell epitopes from studies with immunogens in which the conformation of the epitope recognized by B cells has been determined.

The size of a B-cell epitope is determined by the size of the antigen-binding site on the antibody molecules displayed by B cells. The binding of an antibody to an epitope involves weak noncovalent interactions, which operate only over short distances and therefore depend on complementarity between the antibody's binding site and the epitope to maximize these weak interactions. The size of the epitope recognized by a B cell thus is determined by the size, shape, and amino acid residues of the antibody's binding site.

In the 1950s, Elvin A. Kabat designed experiments to determine the size of the B-cell epitope on the glucose polymer dextran. In these experiments, he measured the ability of short glucose oligomers, varying in length from disaccharides to large oligosaccharides, to inhibit the binding of antidextran antibodies to dextran. Kabat reasoned that an oligomer constituting the entire epitope should be able to totally occupy the antibody's antigen-binding site and thus completely inhibit binding of the antibody to the epitope on the immunogen. As he increased the polymer size from trisaccharide to hexasaccharide, the oligomers showed increasing ability to inhibit the binding of antidextran antibodies to dextran (Figure 4-3). Since heptasaccharides and larger oligosaccharides showed the same inhibitory ability as the hexasaccharide, Kabat predicted that the hexasaccharide best approximated the size of the complete epitope and that additional sugar residues must lie outside the binding site on the antibody molecule. These early studies with small carbohydrate antigens suggested that the antibody's binding site was a cleft of sufficient size to bind six or seven amino acid or sugar residues.

As Ag-Ab complexes were analyzed with x-ray crystallography, a more detailed picture of epitope structure emerged. Smaller ligands such as carbohydrates, nucleic acids, peptides, and haptens were often found to bind to an antibody within a deep concave pocket. Crystallographic analysis of a small octapeptide hormone, called angiotensin II, revealed that the antibody made contact with the octapeptide within a deep and narrow groove of 725 Å^2. Within the groove, the peptide hormone was folded into a compact structure with two turns, which brought both amino and carboxyl termini close together. All eight amino acid residues of the octapeptide were shown to be involved in van der Waals contacts with 14 residues of the antibody groove.

X-ray crystallographic analysis of antibody complexed to globular protein antigens has yielded a very different picture of epitope structure. Analyses of monoclonal antibodies bound to hen egg-white lysozyme or neuraminidase (an envelope glycoprotein of influenza) have revealed that the antibody makes contact with the protein antigen across a large planar face. The interacting face between antibody and epitope has been observed as a somewhat flat to undulating surface in which protrusions on the epitope or antibody are matched by corresponding depressions on the respective antibody or epitope. These studies have revealed that 15–22 amino acids on the surface of the protein antigen make contact with a similar number of residues in the antibody's binding site; the surface area of this large complementary interface is between 650–900 Å^2. For these globular protein antigens, then, the epitope is entirely dependent on the tertiary conformation of the native protein.

Thus a different picture emerges of epitopes in globular protein antigens and in small peptide antigens. An epitope on a globular protein antigen appears to be considerably larger, occupying a more extensive surface area that is dependent on the tertiary structure of the protein; in contrast a smaller ligand, such as angiotensin II, folds into a compact structure that interacts with the antibody within a deep and narrow cleft. In Chapter 5 the nature of the interaction of the epitope with the antigen-binding site of the antibody is examined in more detail.

B-cell epitopes in native proteins generally are hydrophilic amino acids on the protein surface that are topographically accessible to membrane-bound or free antibody. A B-cell epitope must be accessible in order to be able to bind to an antibody. Amino acid sequences that are hidden within the interior of a protein cannot function as B-cell epitopes unless the protein is first denatured. Michael Sela demonstrated the importance of this topographical accessibility in experiments with synthetic branched copolymers in which the accessible amino acids attached to the backbone polypeptide chain were varied. One copolymer, (T,G)-A-L, consisted of a poly-L-lysine backbone with poly D,L-alanine side chains whose N-termini are capped with variable amounts of glutamic acid and/or tyrosine (Figure 4-4). Antibody to (T,G)-A-L reacted largely with the accessible tyrosine and glutamic acid residues at the end of each side chain. Furthermore,

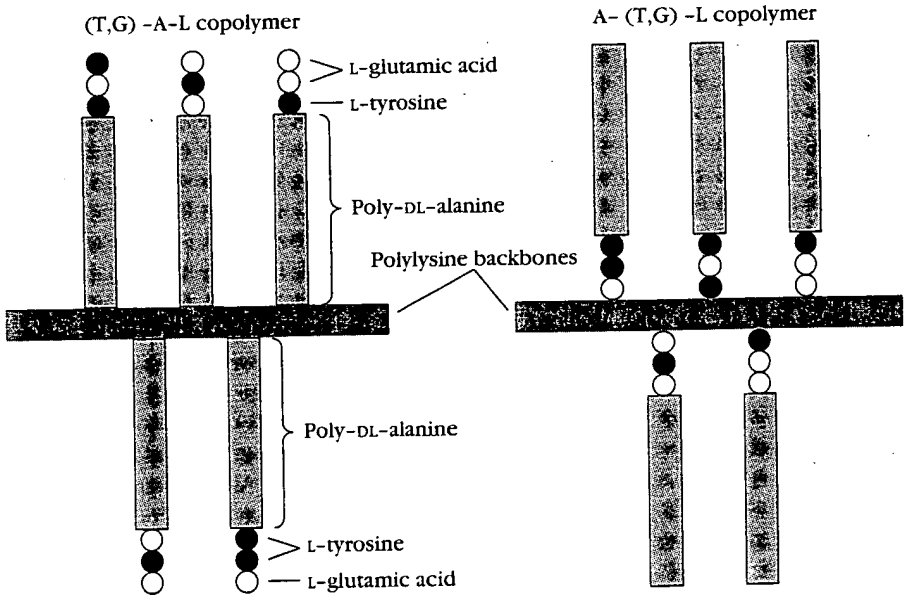


FIGURE 4-4 Antibodies elicited by immunization with the (T,G)-A-L copolymer react largely with the exposed tyrosine and glutamic acid residues. Anti-(T,G)-A-L antibodies do not react with the A-(T,G)-L copolymer in which the tyrosine and glutamic acid residues are buried. [Adapted from M. Sela, 1969, *Science* **166**:1365.]

the related synthetic copolymer A-(T,G)-L, in which poly D,L-alanine residues are in the accessible terminal positions and the glutamic acid and tyrosine residues are in a less accessible position, cannot react with the antibody to (T,G)-A-L.

The entire surface of globular protein antigens is thought to be potentially antigenic. In general, regions that tend to protrude on the surface of the protein are often recognized as epitopes. Because the residues are accessible, they are often hydrophilic. Of the crystallized Ag-Ab complexes analyzed to date, the interface between antibody and antigen possesses numerous complementary protrusions and depressions. Contact is made between 15–22 amino acids and has been shown to involve between 75–120 hydrogen bonds as well as ionic and hydrophobic interactions.

B-cell epitopes can contain sequential or nonsequential amino acids. Epitopes may be composed of *sequential* contiguous residues along the polypeptide chain or *nonsequential* residues from segments of the chain brought together by the folded conformation of the protein. Most antibodies elicited by globular protein antigens bind to the protein only when it is in its native conformation. Because denaturation of such antigens usually results in loss of the topographical structure of their epitopes, antibodies to the native protein fail to bind to the denatured protein.

Sperm whale myoglobin is an example of a protein antigen that contains several sequential epitopes. The three-dimensional structure of this protein has been determined by x-ray crystallography. The molecule has an abundance of α-helical regions and five distinct sequential epitopes, each containing six to eight

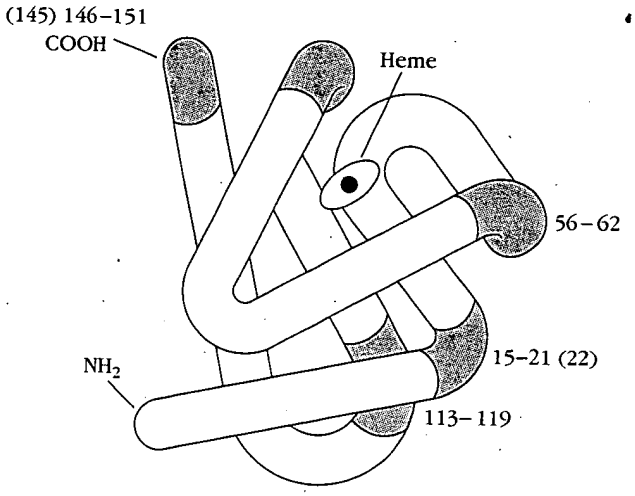
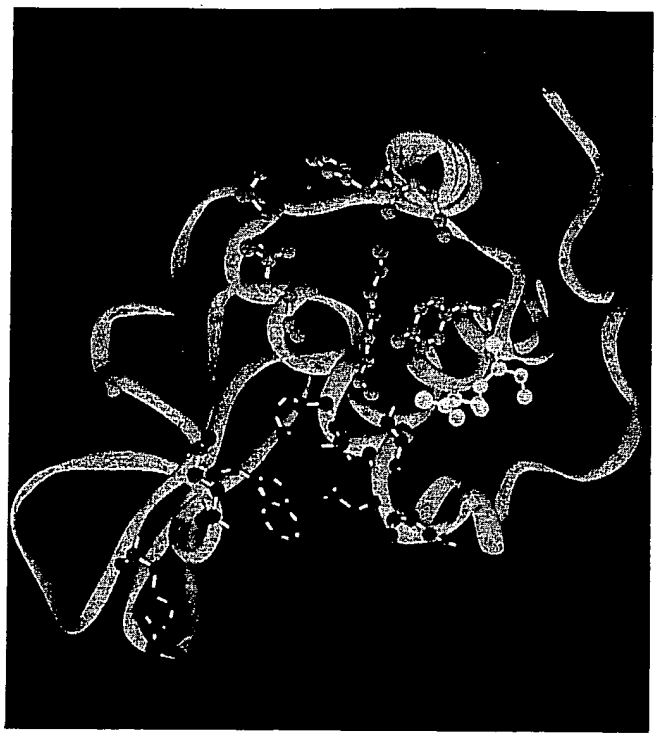
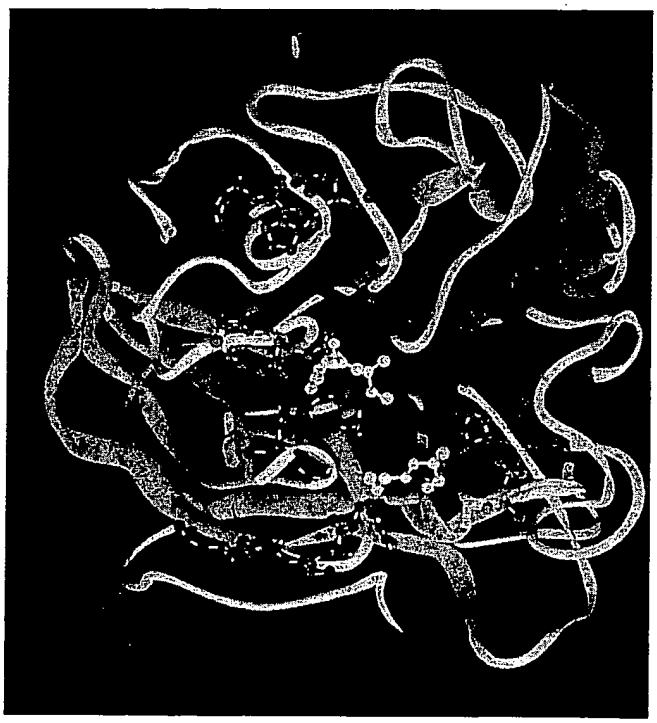


FIGURE 4-5 Diagram of sperm whale myoglobin showing locations of five sequential B-cell epitopes (light purple). [Adapted from M. Z. Atassi and A. L. Kazim, 1978, *Adv. Exp. Med. Biol.* **98**:9.]



(a)



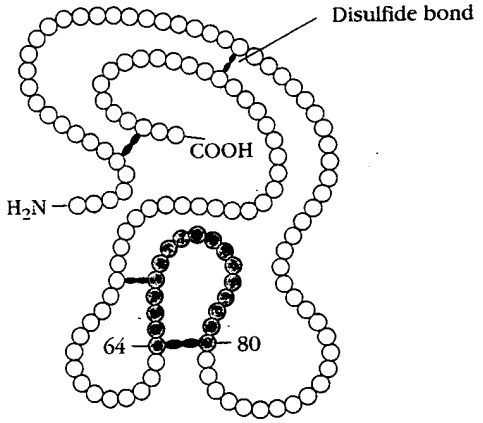
(b)

FIGURE 4-6 Ribbon diagrams of hen egg-white lysozyme (a) and influenza-virus neuraminidase (b) showing the location of one nonsequential epitope (conformational determinant) in each protein. The amino acids that have been shown to contact monoclonal antibody are indicated with shaded balls. [Adapted from W. G. Laver et al. 1990, *Cell* **61**:554.]

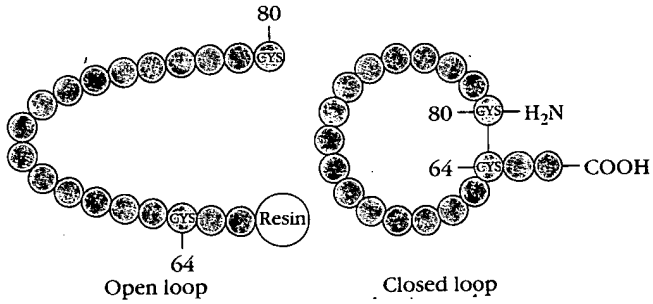
amino acids. Each of these epitopes is on the surface of the molecule at bends between the α-helical regions (Figure 4-5). Recently several additional nonsequential epitopes, or *conformational determinants,* also have been characterized for sperm whale myoglobin. The residues constituting these epitopes are far apart in terms of the primary amino acid sequence but close together in the tertiary structure of the molecule. Such epitopes thus are dependent on the native protein conformation for their topographical structure. The epitopes of hen egg-white lysozyme (HEL) and neuraminidase are well-characterized conformational determinants. Figure 4-6 shows the amino acid residues that make up one epitope of HEL and one epitope of neuraminidase. In each case the epitope is composed of nonsequential amino acids, far apart in the primary amino acid sequence, that have been brought together by the tertiary folding of the protein.

Sequential and nonsequential epitopes generally behave differently when a protein is fragmented or reduced. For example, appropriate fragmentation of sperm whale myoglobin can yield five fragments, each retaining one sequential epitope, as demonstrated by the observation that antibody can bind to each fragment. On the other hand, fragmentation of a protein or reduction of its disulfide bonds often destroys any nonsequential epitopes that it contains. For example, HEL has four intrachain disulfide bonds, which determine the final protein conformation. Antibodies to HEL recognize eight different epitopes, most of which are conformational determinants dependent on the overall structure of the protein. If the intrachain disulfide bonds of HEL are reduced with mercaptoethanol, the conformational determinants are lost and antibody to native HEL will not bind to reduced HEL. The inhibition experiment described in Figure 4-7 also dem-

(a) Hen egg-white lysosome



(b) Synthetic loop peptides



(c) Inhibition of reaction between HEL loop and anti-loop antiserum

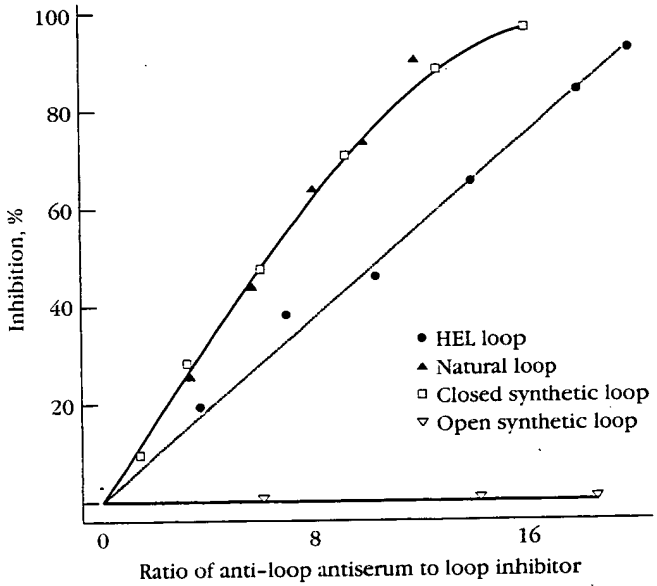


FIGURE 4-7 Experimental demonstration that binding of antibody to conformational determinants in hen egg-white lysozyme (HEL) depends on maintenance of the tertiary structure of the epitopes by intrachain disulfide bonds. (a) Diagram of HEL primary structure in which balls represent amino-acid residues. The loop (purple balls) formed by the disulfide bond between the cysteine residues at positions 64 and 80 constitutes one of the conformational determinants in HEL. (b) Synthetic open- and closed-loop peptides corresponding to the HEL loop epitope. (c) Inhibition of reaction between HEL loop epitope and anti-loop antiserum. Anti-loop antiserum was first incubated with HEL, the natural loop sequence, the synthetic closed-loop peptide, or the synthetic open-loop peptide; the ability of the antiserum to bind the natural loop sequence then was determined. The absence of any inhibition by the open-loop peptide indicates that it does not bind to the anti-loop antiserum. [Adapted from D. Benjamin et al., 1984, *Annu. Rev. Immunol.* **2**:67.]

TABLE 4-5 ANTIGEN RECOGNITION BY T AND B LYMPHOCYTES REVEALS QUALITATIVE DIFFERENCES

		Secondary immune response	
Primary immunization	Secondary immunization	Antibody production	Cell-mediated T_{DTH} response*
Native protein	Native protein	+	+
Native protein	Denatured protein	−	+

* T_{DTH} refers to a subset of $CD4^+$ T_H cells that mediate a type of cell-mediated response known as delayed-type hypersensitivity (see Chapter 15).

onstrates the importance of these disulfide bonds in determining the structure of HEL epitopes.

B-cell epitopes tend to be located in flexible regions of an immunogen and display site mobility. John A. Tainer and his colleagues analyzed the epitopes on a number of protein antigens (myohemerytherin, insulin, cytochrome *c*, myoglobin, and hemoglobin) by comparing the positions of the known B-cell epitopes with the atomic mobility of the same residues. Their analysis revealed that the major antigenic determinants in these proteins generally were located in the most mobile regions. These investigators propose that site mobility of epitopes maximizes complementarity with the antibody's binding site, giving rise to a higher-affinity interaction.

Complex proteins contain multiple overlapping B-cell epitopes. Until recently, it was dogma in immunology that a given globular protein had a small number of epitopes, each confined to a highly accessible region and determined by the overall conformation of the protein. However, it has been shown recently that most of the surface of a globular protein is potentially antigenic. This has been demonstrated by comparing the antigen-binding profiles of different monoclonal antibodies to various globular proteins. For example, when 64 different monoclonal antibodies to BSA were compared for their ability to bind to a panel of 10 different mammalian albumins, 25 different overlapping antigen-binding profiles emerged, suggesting that these 64 different antibodies recognized a minimum of 25 different epitopes on BSA. Similar findings have emerged for other globular proteins, such as myoglobin and HEL. The surface of a protein, then, must present a large number of potential antigenic sites. The subset of antigenic sites on a given protein that is selected by an individual animal is much smaller than the potential antigenic repertoire, and it varies from species to species and even among individual members of a given species. Within a given animal, certain epitopes are recognized as immunogenic, whereas others are not. Furthermore, some epitopes, referred to as *immunodominant,* induce a more pronounced immune response than other epitopes in a particular animal. It is thought that intrinsic topographical properties of the epitope as well as the animal's regulatory mechanisms influence the immunodominance of particular epitopes.

Properties of T-Cell Epitopes

Early studies by P. G. H. Gell and Baruj Benacerraf in 1959 suggested that there is a qualitative difference between the T-cell and the B-cell response to protein antigens. Gell and Benacerraf compared the humoral and cell-mediated responses to a series of native and denatured protein antigens (Table 4-5). They found that if primary immunization was with a native protein, then a secondary antibody response was elicited only with native protein, not with denatured protein. In contrast, the secondary cell-mediated response did not discriminate between native and denatured protein. In other words, a secondary T-cell–mediated response was induced by denatured protein even when the primary immunization had been with native protein. This observation puzzled immunologists until the 1980s, when it became clear that T cells do not recognize soluble native antigen but rather recognize antigen that has been processed and whose peptide fragments are presented in association with MHC molecules. For this reason, destruction of the conformation of a protein by denaturation does not affect its T-cell epitopes.

Because the T-cell receptor does not bind an epitope directly, experimental systems for studying T-cell epitopes must include antigen-presenting cells or target cells that can display the epitope together with an MHC molecule. In some systems, synthetic peptides are first allowed to interact with MHC molecules on antigen-presenting cells, and then T-cell proliferation is measured.

Oligomeric peptides function as T-cell epitopes. S. F. Schlossman synthesized polypeptide polymers containing oligomers of L-lysine residues separated by

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